

Valve Disease

Cardiocyte Cytoskeleton in Patients With Left Ventricular Pressure Overload Hypertrophy

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OBJECTIVES	We sought to determine whether the cardiocyte microtubule network densification characteristic of animal models of severe pressure overload cardiac hypertrophy occurs in human patients.
BACKGROUND	In animal models of clinical entities causative of severe right and left ventricular (LV) pressure overload hypertrophy, increased density of the cellular microtubule network, through viscous loading of active myofilaments, causes contractile dysfunction that is normalized by microtubule depolymerization. These linked contractile and cytoskeletal abnormalities, based on augmented tubulin synthesis and microtubule stability, progress during the transition to heart failure.
METHODS	Thirteen patients with symptomatic aortic stenosis (AS) (aortic valve area = 0.6 ± 0.1 cm ²) and two control patients without AS were studied. No patient had aortic insufficiency, significant coronary artery disease or abnormal segmental LV wall motion. Left ventricular function was assessed by echocardiography and cardiac catheterization before aortic valve replacement. Left ventricular biopsies obtained at surgery before cardioplegia were separated into free and polymerized tubulin fractions before analysis. Midwall LV fractional shortening versus mean LV wall stress in the AS patients was compared with that in 84 normal patients.
RESULTS	Four AS patients had normal LV function and microtubule protein concentration; six had decreased LV function and increased microtubule protein concentration, and three had borderline LV function and microtubule protein concentration, such that there was an inverse relationship of midwall LV fractional shortening to microtubule protein.
CONCLUSIONS	In patients, as in animal models of severe LV pressure overload hypertrophy, myocardial dysfunction is associated with increased microtubules, suggesting that this may be one mechanism contributing to the development of congestive heart failure in patients with AS. (J Am Coll Cardiol 2001;37:1080-4) © 2001 by the American College of Cardiology

The transition from compensated cardiac hypertrophy to decompensated cardiac failure is one of the central problems in clinical cardiology. Cardiac hypertrophy after hemodynamic overloads fails to be functionally compensatory either when the load increase exceeds the inherent growth capacity of the terminally differentiated cardiac muscle cell, or cardiocyte, to renormalize stress or when the intrinsic contractile function per unit mass of hypertrophied myocardium is less than that of normal myocardium. Thus, cardiac compensation for an increased load may be imperfect because of either quantitative or qualitative defects of hypertrophied myocardium.

Our search for the basis of the qualitative defects of hypertrophied myocardium, which are thought to underlie the frequent deterioration of initially compensatory cardiac hypertrophy into the congestive heart failure state, has

resulted in two novel findings (1,2). First, the contractile abnormalities of the cardiocyte hypertrophying in response to a severe pressure overload are accounted for to a remarkable degree by increased density of the cellular microtubule network, which imposes a viscous load on the shortening sarcomeres during contraction (3), with normal contractile function being restored when the microtubules are depolymerized. Second, increased microtubules, as well as nonpolymerized alpha-beta-tubulin heterodimers, are present as soon as hypertrophy is fully established, and this increase is persistent and progressive thereafter (4,5). These findings, which obtain both in the right and in the left ventricle (LV) of multiple species at the levels of isolated cells (1,2,6,7), excised tissue (8) and the intact heart in vivo (9), are based on altered transcriptional regulation of the beta-tubulin multigene family (10) and of the predominant microtubule-associated protein (MAP) of the heart, MAP 4 (11).

A central goal of this work has been to determine whether this cytoskeletal abnormality has a role in clinical heart disease. Thus, we have proceeded in animal models from cells, to tissue, to the heart of the intact animal and then in clinical studies to patients with pressure overload hypertrophy. This study, which constitutes the step from the intact animal to patients, was designed to address the question of

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Abbreviations and Acronyms

AS	= aortic stenosis
ASE	= American Society of Echocardiography
LV	= left ventricle, left ventricular
MAP	= microtubule-associated protein

whether increased cardiac microtubules are characteristic of pressure overload hypertrophy in man.

METHODS

Patient selection and procedures. Thirteen symptomatic patients with isolated valvular aortic stenosis (AS), normal segmental LV wall motion and no significant aortic insufficiency or coronary artery disease were studied; surgical timing was decided by the referring physician. Two control patients had aortic reconstruction for Marfan's syndrome; they had no cardiac pathology as assessed by echocardiography and cardiac catheterization. Patient recruitment, echocardiography, cardiac catheterization and aortic valve surgery with LV biopsy occurred at Stanford University. The LV function data and biopsies were analyzed at the Medical University of South Carolina.

Analysis of LV function. Left ventricular function was evaluated as follows before surgery. Cardiac catheterization was used to measure LV pressure and aortic valve gradient and area, and echocardiography was used to measure LV dimension, thickness, shortening and wall stress. Left ventricular function was characterized in terms of the midwall fractional shortening versus mean systolic stress relationship. Midwall shortening was calculated from the two-shell cylindrical model (12). Mean LV systolic stress (σ) was calculated using a cylindrical model as circumferential mid-wall stress (12):

$$\sigma = Pa^2 \frac{[1 + (b^2/r^2)]}{(b^2 - a^2)}$$

where σ was calculated as the average of stress values measured at the three times of aortic valve opening, peak systole and end systole and where P is LV pressure, a is LV endocardial radius, b is LV epicardial radius, and r is LV midwall radius. The rationale for the usage of average stress values is the fact that the most important determinant of ejection performance is stress throughout systole, when ejection is occurring, rather than stress measured solely at the end of ejection.

To calculate peak systolic stress, P was measured as peak systolic LV pressure at the time of cardiac catheterization and confirmed at echocardiography by sphygmomanometer measurement of systolic arterial pressure plus the Doppler-derived aortic gradient. The values for a , b and r were calculated from dimensions at one-third shortening. This method has been used in clinical (13) and experimental (14) studies of LV function and has been validated in patients with AS (15).

To calculate end-systolic stress, P was measured as the aortic diastolic notch pressure at cardiac catheterization and confirmed during echocardiography by sphygmomanometer measurement as the mean arterial pressure (16). The values for a , b and r were measured from end-systolic dimensions defined by the American Society of Echocardiography (ASE) criteria (17).

To calculate wall stress at the instant of aortic valve opening, P was measured as the aortic anacrotic notch pressure at cardiac catheterization and confirmed during echocardiography by sphygmomanometer measurement as the mean arterial pressure. The values for a , b and r were measured from end-diastolic dimensions (the LV remains isovolumic between end diastole and aortic valve opening) defined by the ASE criteria (17).

Mean systolic wall stress during ejection was, thus, approximated by calculating the area under these three stress-time coordinates at aortic valve opening, at peak systole and at end systole (18).

A cohort of 84 patients not otherwise part of this study was used to define the normal midwall fractional shortening versus mean systolic stress relationship, given as the mean \pm 95% prediction interval by the solid and dashed lines, respectively, in Figure 1. These 84 patients (age: 59 ± 15 years; gender: 44 female/42 male) will be seen in comparison with the patients with AS defined in Table 1 to be slightly younger and to have the same gender distribution. They were studied via outpatient echocardiography and sphygmomanometer-determined arterial blood pressure in the course of standard care, had no evidence of cardiac disease and had normal absolute and relative LV wall thickness, size and function. For the patients with AS and two control patients reported in this study, LV function was characterized with reference to the relationship thus defined in these 84 normal patients.

Analysis of microtubule protein. After instituting cardiopulmonary bypass, but before instituting cold cardioplegia, an epicardial wedge biopsy (25 to 50 mg) was taken from the LV free wall of each of the 15 patients. The biopsy was immediately divided into free tubulin and polymerized tubulin (microtubule) protein fractions that were prepared as follows. Each biopsy specimen was homogenized in 1 ml of microtubule stabilizing buffer (19) and centrifuged at 100,000 g , 25°C for 15 min. The supernatant was saved as the free tubulin fraction, and the pellet was resuspended at 0°C in 1 ml of microtubule depolymerization buffer (19); after 1 h at 0°C, it was centrifuged at 100,000 g , 4°C for 15 min, and the supernatant was saved as the polymerized tubulin fraction. Protease inhibitors (20) were used throughout. For the subsequent 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis, an equal amount of free tubulin protein as determined by a bicinchoninic acid assay (Pierce, Rockford, Illinois) was loaded in the first lane for each biopsy sample, and an equivalent volume of the polymerized tubulin fraction was loaded in the second lane. The samples were transferred to polyvinylidene difluoride

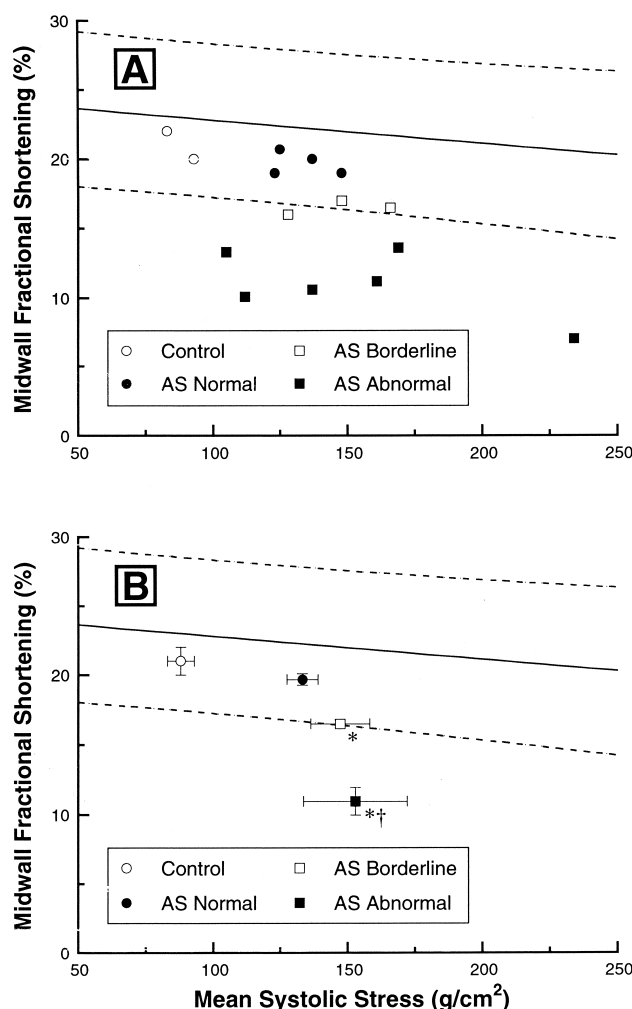


Figure 1. Left ventricular function in control patients and in patients with AS. (Both panels) The solid and parallel dashed lines define the relationship between midwall fractional shortening and mean systolic stress, as well as its 95% prediction interval, and calculated using a least squares linear regression analysis for 84 patients found not to have cardiac pathology. The shortening-stress relationship was characterized as noted in the "Methods" section identically for both the 84 patients used to generate the regression analysis and for the study patients having AS. (Panel A) Individual values for the 15 patients in this study. (Panel B) Summary data for these same patients grouped by functional status. AS = aortic stenosis.

membranes (35 V, 75 min) and probed with a 1:3,000 dilution of a beta-tubulin monoclonal antibody (Amersham, Arlington Heights, Illinois). Bound antibody was visualized

Table 1. Characteristics of Patients With Aortic Stenosis

Characteristics	Values
Age (yr)	69 ± 5
Gender (M/F)	7/6
Etiology, calcific/bicuspid/rheumatic	7/4/2
Aortic valve area (cm ²)	0.6 ± 0.1
Mean aortic valve gradient (mm Hg)	47 ± 10
Symptoms, CHF/angina/syncope	11/7/2
NYHA Class, I/II/III/IV	1/5/6/1

Values are either mean ± SEM or number of patients having a given characteristic. CHF = congestive heart failure; NYHA class = New York Heart Association functional class.

Table 2. LV Characteristics Versus Microtubule Protein

Group	Midwall Fractional Shortening (%)	Mean Systolic Stress (g/cm ²)	Microtubule Protein (I.O.D.)
Control (n = 2)	21.0 ± 1.0	88.0 ± 5.0	1.18 ± 0.41
AS Normal (n = 4)	19.7 ± 0.4	133.3 ± 5.8	0.67 ± 0.24
AS Borderline (n = 3)	16.5 ± 0.3	147.3 ± 11.0	6.33 ± 2.89
AS Abnormal (n = 6)	11.0 ± 1.0	153.0 ± 19.3	19.31 ± 5.00

Values are mean ± SEM. For each of the three LV characteristics shown here, there was a significant ($p \leq 0.05$) trend in the data, as shown by the Spearman rank correlation coefficient, associated with worsening clinical state.

AS = aortic stenosis; I.O.D. = integrated optical density (arbitrary units) from the immunoblots; LV = left ventricular.

with a horseradish peroxidase-conjugated secondary antibody (Vector Labs, Burlingame, California) and enhanced chemiluminescence (NEN Life Science Products, Boston, Massachusetts). In all cases, a single band at 55 kDa having the same mobility as bovine brain beta-tubulin was detected. Densitometric quantification of the immunoblots, using a concurrently run bovine brain beta-tubulin standard, was carried out precisely as before (1).

Statistical analysis. Values are given as mean ± SEM for each data group. Statistical comparisons, defined in the legends of the figures and tables wherein they were employed, were said to show a significant difference at the $p \leq 0.05$ level.

RESULTS

Characteristics of the patients. The major features of the 13 patients with valvular AS are summarized in Table 1. Medications included diuretics in five patients, beta-adrenergic blocking agents in four patients, calcium channel blockers in four patients and angiotensin-converting enzyme inhibitors in two patients. No patient suffered a biopsy-related complication.

LV function. Figure 1A shows individual values for the midwall fractional shortening versus mean systolic stress relationship for the 2 control patients and 13 patients with AS, defined in terms of 84 patients having normal cardiac function. The 13 aortic stenosis patients self-segregated functionally into three groups: 4 with normal LV function (AS Normal), 3 with borderline abnormal LV function (AS Borderline) and 6 with distinctly abnormal LV contractile function (AS Abnormal). The summary data given in Figure 1B and Table 2 show that there is a progressive decrement in midwall left ventricular fractional shortening when proceeding from "AS Normal" through "AS Borderline" to "AS Abnormal."

Microtubule protein. The upper panel of Figure 2 is an immunoblot of the free and polymerized tubulin fractions from the myocardial biopsy of one patient from each of the four categories identified in Figure 1. To facilitate visual comparisons, protein loading was adjusted so that the amount of free tubulin loaded for each patient (lanes #1) was equal, and the volume ratio of the free to polymerized (lanes #2) tubulin fractions loaded was held constant. Thus, it is clear that while "Control" and "AS Normal" are

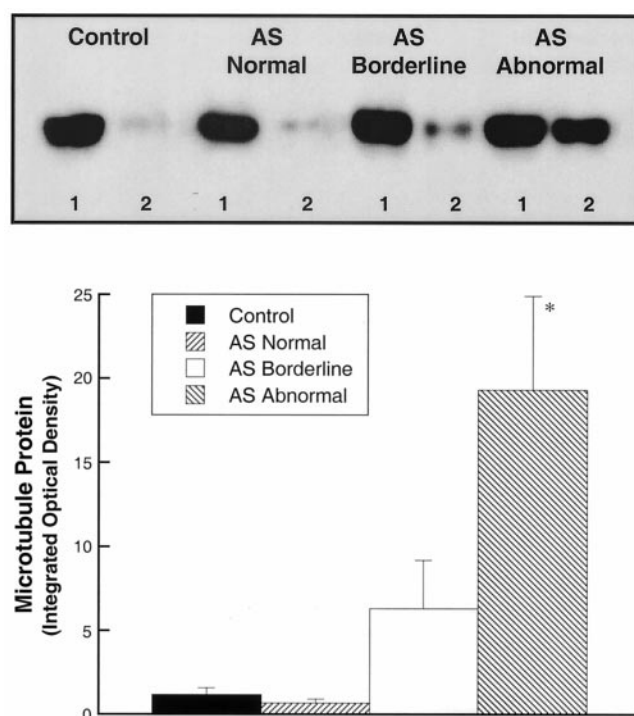


Figure 2. Representative and summarized myocardial tubulin data for normal patients and patients with AS. **(Top)** Immunoblot prepared from left ventricular biopsies from one patient from each of the four study groups defined in Figure 1. For comparison of the ratio of free heterodimer (**lanes #1**) to polymerized microtubule (**lanes #2**) tubulin in the four biopsies, an equal amount of tubulin protein was loaded for each of the free tubulin lanes, and an equal volume of the polymerized tubulin (microtubule) fraction was loaded for each of the polymerized tubulin lanes. **(Bottom)** Summary semiquantitative tubulin data for all of the patients in the four groups defined in Figure 1; the values were obtained from immunoblots wherein known quantities of concurrently run pure beta-tubulin were used as a standard, and the integrated optical density of the standards was compared with that of the unknown samples. Statistical analysis by one factor analysis of variance, which showed a significant ($p < 0.05$) F-ratio, was followed by a Fisher protected least significant difference post-hoc test. * $p < 0.05$ for difference from the value for the Control group. AS = aortic stenosis.

equivalent, there is a progressive increase in the ratio of polymerized/free tubulin when proceeding from "AS Normal" through "AS Borderline" to "AS Abnormal." The lower panel of Figure 2 that provides summary data for the immunoblots from all 15 patients extends this result in semiquantitative terms. As shown here and in Table 2, microtubule-incorporated tubulin, as a fraction of total protein, shows a significant, progressive increase when proceeding from "AS Normal" through "AS Borderline" to "AS Abnormal."

The relationship of LV function to microtubule protein, given in Figure 3, is inverse. Thus, in proceeding from "AS Normal" through "AS Borderline" to "AS Abnormal," the successive decrements in LV systolic contractile function are associated with successive increments in microtubule protein concentration.

DISCUSSION

The results of this study are summarized in Table 2. In those patients with AS having an increase in the concen-

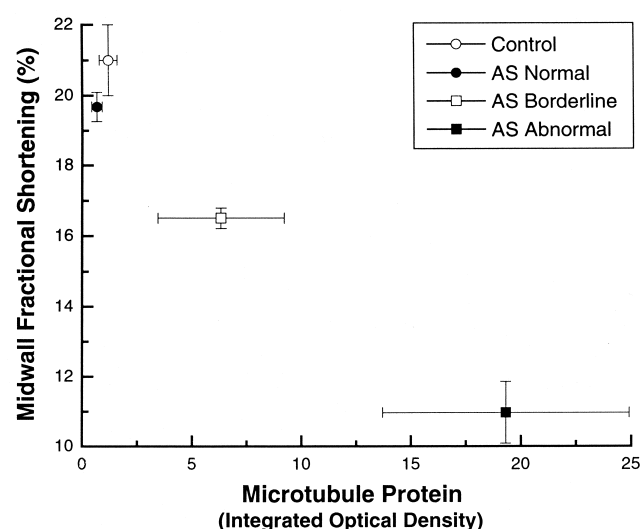


Figure 3. The relationship of left ventricle contractile function to left ventricle microtubule protein for all of the patients in the four groups defined in Figure 1. AS = aortic stenosis.

tration of myocardial microtubule protein, there is a corresponding decrease in ventricular function as measured by midwall fractional shortening, associated with significantly increasing LV wall stress. In the clinical setting, a modality having an acceptable therapeutic index does not exist for removing the microtubules from interphase cells, so that we could not establish in these patients, as we have done in experimental models, a direct cause-and-effect relationship between increased myocardial microtubules and decreased myocardial contractile function. Nonetheless, these data do allow us to conclude that, in man, as in animal models of chronic, severe, pressure overload hypertrophy, myocardial dysfunction is associated with an increase in the density of the cardiocyte microtubule network, so that this cytoskeletal alteration may be one mechanism that causes the development of clinical myocardial failure in this specific hemodynamic setting.

Prevalence of the finding. The specificity of this alteration of the extra-myofilament cytoskeleton to severe pressure overload hypertrophy causing increased ventricular wall stress bears emphasis. In our initial reports of this phenomenon (1,2), the data from an animal model with severe pressure overload hypertrophy were compared with those from a closely related animal model having severe volume overload hypertrophy of an equivalent degree and duration. Myocardial contractile dysfunction and the associated microtubule change found therein to be causative of that contractile dysfunction were unique to the pressure-overloaded myocardium; neither was present to any degree whatsoever in the volume-overloaded myocardium. In subsequent work by others and us described elsewhere (8), the specificity of increased microtubule network density to the hemodynamic setting noted above has been further established. Thus, while these findings apply to a very important clinical problem, they apply, based on studies to date, solely to that particular problem.

Validation of the finding. The validity of any newly proposed pathophysiological mechanism of disease may be tested in terms of the postulates originally framed by Robert Koch (21). While, again, we are unable for practical reasons to do this in humans, we have tested this new mechanism in cell, tissue and whole animal experimental models of human disease. In accordance with Koch's first and second postulates, the microtubule increase is absent in even extensive pressure overload cardiac hypertrophy so long as muscle function remains normal; rather, increased microtubules develop solely in dysfunctional hypertrophied muscle and only at the time that such dysfunction appears, at which time microtubule depolymerization restores normal contractile function (6). In accordance with Koch's third postulate, chemical and physical agents, which increase microtubules independent of hemodynamic input, were shown in these *in vitro* and *in vivo* studies (1-11,22) to reproduce the contractile and cytoskeletal abnormalities seen with severe pressure overload cardiac hypertrophy.

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